International Application No PCT/US88/00058

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Science, Vol. 229, no. 4712, issued 1985, 02 August (Washington, D.C., USA) J.L. MARX. "Antibodies Made To Order", pp 455-456. See entire document.	
Y	Jour. Immunol., vol. 125, no. 4, issued 1980 October (Baltimore, Maryland USA) P. Stashenko et al "Characterization Of A Human B-lymphocyte Specific Antigen", See page 1678, column 2, lines 5-16.	1-29

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers because they relate to subject matter [12] not required to be searched by this Authority, namely:

2.☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/00058

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 39/395;C07H 19/06; See Continuation sheet 1.
U.S.Cl.: 424/88;435/7,68,172.3,240.2,948; See Continuation.

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/1.1,88;435/7,68,172.3,240.2,948; 436/518,519, 536,548;530/387,388,391,808;535/27;935/50,70,72
INT.CL.	A61K 39/395

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

BIOSIS "BP35","CD20 AND CHIMERIC"

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	EP, A, 0,125,023 (CABILLY ET AL) 14 November 1984. See page 3, line 19- page 4, line 2; page 8, lines 29-34; page 11, line 26 - page 12, line 12; page 14, line 24 - page 19, line 8; page 21, lines 15-33; page 28, line 16 - page 29, line 5; page 32, lines 25-35.	1-27
Y	Proc. Natl. Acad. Sci. USA Vol. 82, no. 6, issued 1985 March (Washington, D.C., USA), E.A. CLARK ET AL, "Role Of The Bp 35 Cell Surface Polypeptide In Human B-cell Activation", pp 1766-1770. See page 1766, column 2, lines 1-6.	1-29
Y	EP, A, 0,173,494 (MORRISON ET AL) 05 March 1983. See page 1, lines 19-27; page 7, line 16 - page 8, line 5; page 11, line 1 - page 13, line 2; page 13, line 35 - page 14, line 18.	1-29

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

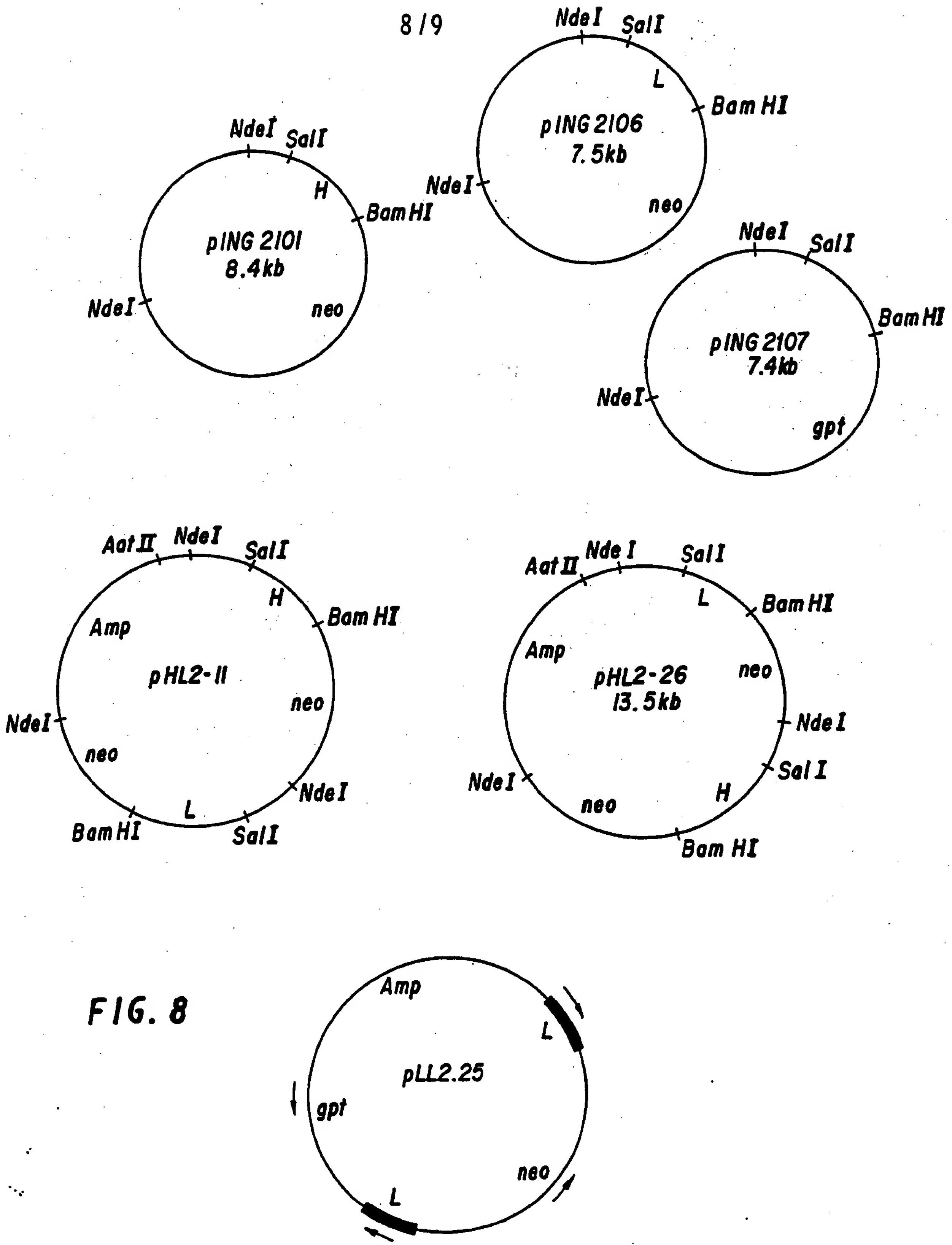
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
14 FEBRUARY 1988	23 MAR 1988
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	David A. Saunders David A. Saunders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NdeI
SalI
NdeI
neo
H
BamHI
Amp
L
pLL2.25
gpt
neo
L

FIG. 8

2H7 LIGHT CHAIN VARIABLE SEQUENCE

```
                                                                 leader peptide
                        met asp phe gln val gln ile phe ser phe leu leu
C23CCCAAAATTCAAAGACAAAATG GAT TTT CAA GTG CAG ATT TTC AGC TTC CTG CTA
            GTC            SalI primer
                                     | FRI
ile ser ala ser val ile ile ala arg gly gln ile val leu ser gln ser
ATC AGT GCT TCA GTC ATA ATT GCC AGA GGA CAA ATT GTT CTC TCC CAG TCT

                                                                FRI |
pro ala ile leu ser ala ser pro gly glu lys val thr met thr cys arg
CCA GCA ATC CTG TCT GCA TCT CCA GGG GAG AAG GTC ACA ATG ACT TGC AGG

CDRI                            CDRI |FR2
ala ser ser ser val ser tyr met his trp tyr gln gln lys pro gly ser
GGC AGC TCA AGT GTA AGT TAC ATG CAC TGG TAC CAG CAG AAG CCA GGA TCC
                                     KpnI ↑                  ↑BamHI
                        FR2| CDR2                   CDR2| FR3
ser pro lys pro trp ile tyr ala pro ser asn leu ala ser gly val pro
TCC CCC AAA CCC TGG ATT TAT GCC CCA TCC AAG CTG GCT TCT GGA GTC CCT

ala arg phe ser gly ser gly ser gly thr ser tyr ser leu thr ile ser
GCT CGC TTC AGT GGC AGT GGG TCT GGG ACC TCT TAC TCT CTC ACA ATC AGC

                                            FR3| CDR 3
arg val glu ala glu asp ala ala thr tyr tyr cys gln gln trp ser phe
AGA GTG GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAG CAG TGG AGT TTT
                              Jκ5
        CDR3 | FR4                                  FR4
asn pro pro thr phe gly ala gly thr lys leu glu leu lys
AAC CAA CCC ACG TTC GGT GCT GGG ACC AAG CTG GAG CTG AAA
                                          T       JκHindIII primer
```

FIG. 6

pGMH6 Human $C_{\gamma}1$ constant domain module

G GTC ACC GTC TCT TCA | GCC TCC ACC AAG GGC CCA TCG GTC TTC -

J_H

Bst E II

Apa I

pGML60 Human C_{κ} constant domain module

GAT CAT CTC CCT CTC ACT TTC GGC GGA GGG ACC AAG GTG GAG ATG AAA | -

$J_{\kappa}4$

C-T

Hind III

J_{κ} Hind III

FIG. 4

Ig heavy chain J-C region

human heavy chain J regions

J | CH1

```
JH1    GCTGAATACTTCCAGCACTGGGGCCAGGGCACCCTGGTCACCGTCTCCTCAG
JH2   CTACTGGTACTTCGATCTCTGGGGCCGTGGCACCCTGGTCACTGTCTCCTCAG
JH3         ATGCTTTTGATGTCTGGGGCCAAGGGACAATGGTCACCGTCTCTTCAG
JH4         ACTACTTTGACTACTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAG
JH5       ACACTGGTTCGACTCCTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAG
JH6 AT(TAC)5 GGTATGGACGTCTGGGGGCAAGGGACCACGGTCACCGTCTCCTCAG
Consensus         TCGACCTCTGGGGCCAAGGAACCCTGGTCACCGTCTCCTCAG
```

mouse heavy chain J regions

J | CH1

```
JH1    TACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCAG
JH2           TACTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAG
JH3         CCTGGTTTGCTTACTGGGGCCAAGGGACTCTGGTCACTGTCTCTGCAG
JH4    TACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCAG
Consensus         TTTGACTACTGGGGCCAAGGGACCACGGTCACCGTCTCCTCAG
```

Ig light chain J-C region

human Kappa J region

J | C

```
JK1   GGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAAC
JK2   ACACTTTTGGCCAGGGGACCAAGCTGGAGATCAAAC
JK3   TCACTTTCGGCCCTGGGACCAAAGTGGATATCAAAC
JK4   TCACTTTCGGCGGAGGGACCAAGGTGGAGATCAAAC
JK5   TCACCTTCGGCCAAGGGACACGACTGGAGATTAAAC
Consensus TTCGGCCAAGGGACCAAGGTGGAGATCAAAC
```

mouse Kappa J region

J | C

```
JK1 TGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAAC
JK2 TACACGTTCGGAGGGGGGACCAAGCTGGAAATAAAAC
JK3 TTCACATTCAGTGATGGGACCAGACTGGAAATAAAAC
JK4 TTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAAAC
JK5 CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAAC
Consensus TTCGGTGGGGGGACCAAGCTGGAAATAAAAC
UIG(MJK)          3'TGGTTCGACCTTTATTTTG5'
```

human Lambda pseudo J region

J | C

```
JPSL1 CACATGTTTGGCAGCAAGACCCAGCCCACTGTCTTAG
```

mouse Lambda J region

J | C

```
JL1 TGGGTGTTCGGTGGAGGAACCAAACTGACTGTCCTAG
JL2 TATGTTTTCGGCGGTGGAACCAAGGTCACTGTCCTAG
JL3 TTTATTTTCGGCAGTGGAACCAAGGTCACTGTCCTAG
Consensus TTCGGCGGTGGAACCAAGGTCACTGTCCTAG
```

FIG.2

23. The process of any of claims 20, 21 or 22 wherein said host is prokaryotic.

24. The process of any of claims 20, 21 or 22 wherein said host is eukaryotic.

25. An immunoassay method for the detection of a 35 kDa polypeptide normally expressed on the surface of B cells in a sample, which comprises:

contacting said sample with the antibody of claim 17 and

detecting whether said antibody binds to said antigen.

26. An _in vivo_ or _in vitro_ imaging method to detect an antigen comprising a 35 kDa polypeptide normally expressed on the surface of B cells which comprises contacting said antigen with the labelled antibody of claim 18 and detecting said antibody.

27. A method of killing cells carrying an antigen thereon, which antigen comprising a 35 kDa polypeptide normally expressed on the surface of B cells which comprises:

contacting said cells with the antibody of claim 17.

28. The method of claim 27 wherein said killing occurs by complement mediated lysis of said cells.

29. The method of claim 27 wherein said killing occurs by ADCC.

10. The molecule of claim 9 wherein said vehicle is a plasmid.

11. A prokaryotic host transformed with the molecule of claim 4.

12. The host of claim 11 which is a bacterium.

13. A eukaryotic host transfected with the molecule of claim 4.

14. The host of claim 13 which is yeast or a mammalian cell.

15. A heavy immunoglobulin chain comprising a constant human region and a variable region having specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells.

16. A light immunoglobulin chain comprising a constant human region and a variable region having specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells.

17. A chimeric antibody molecule comprising two light chains and two heavy chains, each of said chains comprising a constant human region and a variable region having specificity to a 35 kDa polypeptide (Bp35(CD20)) expressed on the surface of human B cells.

18. The antibody of claim 17 in detectably labelled form.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 39, line 11 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [4]

12301 Parklawn Drive
Rockville, Maryland 20852
United States of America

Date of deposit [5]	Accession Number [6]
02 January 1987	HB 9303

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C273 (mouse SP2/0 hybridoma cells carrying plasmids pING 2101 and pING 2106)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer) Annette Short

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

The present invention is not to be limited in scope by the cell lines deposited since the deposited embodiment is intended as a single illustration of one aspect of the invention and all cell lines which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

previously described for mouse monoclonal anti-carcinoma antibody L6, which can mediate ADCC, as well as complement-mediated cytoxicity, CDC. The techniques used and the data described for the L6 antibody have been previously described. Hellstrom, et al., Proc. Natl. Acad Sci. U.S.A. 83: 7059-7063 (1986).

Chimeric 2H7, but not mouse 2H7 antibody, will be able to mediate both ADCC and CDC against human B lymphoma cells. Thus a hybridoma producing a non-functional mouse antibody can be converted to a hybridoma producing a chimeric antibody with ADCC and CDC activities. Such a chimeric antibody is a prime candidate for the treatment or imaging of B cell disorders, such as leukemias, lymphomas, and the like.

This invention therefore provides a method for making biologically functional antibodies when starting with a hybridoma which produces antibody which has the desired specificity for antigen but lacks biological effector functions such as ADCC and CDC.

Conclusions.

The results presented above demonstrate that the chimeric 2H7 antibody binds to (Bp35(CD20)) antigen positive human B cells to approximately the same extent as the mouse 2H7 monoclonal antibody. This is significant because the 2H7 antibody defines a surface phosphoprotein antigen (Bp35(CD20)), of about 35,000 daltons, which is expressed on the cells of B cell lineage. The 2H7 antibody does not bind detectably to various other cells such as fibroblasts, endothelial cells, or epithelial cells in the major organs or the stem cell precursors which give rise to B cells.

e. The conductance of the concentrated supernatant was adjusted to 5.7-5.6 mS/cm CDM 80 radiometer and the pH was adjusted to 8.0.

f. The supernatant was centrifuged at 2000xg, 5 min., and then loaded onto a 40 ml DEAE column, which was preequilibrated with 10mM sodium phosphate, pH8.0.

g. The flow through fraction was collected and loaded onto a 1ml protein A-Sepharose (Sigma) column preequilibrated with 10mM sodium phosphate, pH8.0.

h. The column was washed first with 6ml 10mM sodium phosphate buffer pH 8.0, followed by 8ml 0.1M sodium citrate pH 3.5, then by 6ml 0.1M citric acid (pH 2.2). Fractions of 0.5ml were collected in tubes containing 50ul 2M Tris base (Sigma).

i. The bulk of the IgG was in the pH 3.5 elution and was pooled and concentrated over Centricon 30 (Amicon Corp.) to approximately .06ml.

j. The buffer was changed to PBS (10mM sodium phosphate pH 7.4, 0.15M NaCl) in Centricon 30 by repeated diluting with PBS and reconcentrating.

k. The IgG solution was then adjusted to 0.10ml and bovine serum albumin (Fraction V, U.S. Biochemicals) was added to 1.0% as a stabilizing reagent.

(9) <u>Chimeric 2H7 Antibody, Like the Mouse 2H7 Antibody, Specifically Binds to Human B Cells</u>.

First, the samples were tested with a binding assay, in which cells of both an 2H7 antigen-positive and an 2H7 antigen-negative cell line were incubated with standard mouse monoclonal antibody 2H7 with chimeric 2H7 antibody derived from the cell culture super-

was previously combined with a mouse V_H gene module to form the chimeric expression plasmid pING2012E (Figure 7C).

(5) Chimeric 2H7 Expression Plasmids.

A 2H7 chimeric heavy chain expression plasmid was derived from the replacement of the V_H module of pING2012E with the V_H cDNA modules to give the expression plasmid pING2101 (FIGURE 7B). This plasmid directs the synthesis of chimeric 2H7 heavy chain when transfected into mammalian cells.

For the 2H7 light chain chimeric gene, the SalI to HindIII fragment of the mouse V_K module was joined to the human C_K module by the procedure outlined in FIGURE 7A, forming pING2106. Replacement of the neo sequence with the *E. coli* gpt gene derived from pSV2-gpt resulted in pING2107, which expresses 2H7 chimeric light chain and confers mycophenolic acid resistance when transfected into mammalian cells.

The inclusion of both heavy and light chain chimeric genes in the same plasmid allows for the introduction into transfected cells of a 1:1 gene ratio of heavy and light chain genes leading to a balanced gene dosage. This may improve expression and decrease manipulations of transfected cells for optimal chimeric antibody expression. For this purpose, the DNA fragments derived from the chimeric heavy and light chain genes of pING2101 and pING2106 were combined into the expression plasmids pHL2-11 and pHL2-26 (FIGURE 8). The pHL2-11 and pHL2-26 plasmids each contain a selectable neo^R marker and separate transcription units for each chimeric gene, each gene including a mouse heavy chain enhancer.

The modifications and V-C joint regions of the 2H7 chimeric genes are summarized in FIGURE 9.

region probe. From the 2H7 library, several clones were isolated. A second screen with a 5' J_K5 specific probe identified the 2H7 (J_K5) light-chain clones. Heavy chain clones of 2H7 were generated by priming the poly(A+) RNA with the UIGH(BstEII) oligonucleotide (see Figure 3), and identified by screening with the UIGH(BstEII) oligonucleotide.

The heavy and light chain genes or gene fragments from the V_H and V_K cDNA clones pH2-11 and pL2-12 were inserted into M13 bacteriophage vectors for nucleotide sequence analysis. The complete nucleotide sequences of the variable region of these clones were determined (FIGURES 5 and 6) by the dideoxy chain termination method. These sequences predict V region amino acid compositions that agree well with the observed compositions, and predict peptide sequences which have been verified by direct amino acid sequencing of portions of the V regions.

The nucleotide sequences of the cDNA clones show that they are immunoglobulin V region clones as they contain amino acid residues diagnostic of V domains (Kabat et al., Sequences of Proteins of Immunological Interest; U.S. Dept of HHS, 1983).

The 2H7 V_H has the J_H1 sequence. The 2H7 V_L is from the V_K-KpnI family (Nishi et al. Proc. Nat. Acd. Sci. USA 82:6399, 1985), and uses J_K5. The cloned 2H7 V_L predicts an amino acid sequence which was confirmed by amino acid sequencing of peptides from the 2H7 light chain corresponding to residues 81-100. The cloned 2H7 V_H predicts an amino acid sequence confirmed also by peptide sequencing, namely residues 1-12.

phenolic acid (Calbiochem) was at 6 ug/ml plus 0.25 mg/ml xanthine.

Assays for Immunoglobulin Synthesis and Secretion

Secreted immunoglobulin was measured directly from tissue culture cell supernatants. Cytoplasmic protein extract was prepared by vortexing 10^6 cells in 160 ul of 1% NP40, 0.15 M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.6 and leaving the lysate at 0^oC, 15 minutes, followed by centrifugation at 10,000 x *g* to remove insoluble debris.

A double antibody sandwich ELISA (Voller, A. et al., in Manual of Clinical Immunology, 2nd Ed., Eds. Rose, N. and Friedman, H., pp. 359-371, 1980) using affinity purified antisera was used to detect specific immunoglobulins. For detection of human IgG, the plate-bound antiserum is goat anti-human IgG (KPL, Gaithersburg, Maryland) at 1/1000 dilution, while the peroxidase-bound antiserum is goat anti-human IgG (KPL or Tago, Burlingame) at 1/4000 dilution. For detection of human immunoglobulin kappa, the plate-bound antiserum is goat anti-human kappa (Tago) at 1/500 dilution, while the peroxidase-bound antiserum is goat anti-human kappa (Cappel) at 1/1000 dilution.

EXAMPLE 1

A Chimeric Mouse-Human Immunoglobulin with Specificity for a Human B Cell Surface Antigen

(1) Antibody 2H7.

The 2H7 mouse monoclonal antibody (gamma 2b, kappa) recognizes a human B-cell surface antigen, (Bp35(CD20)) Clark, E.A., et al., Proc. Natl. Acad. Sci., U.S.A. 82:1766 (1985)). The (Bp35(CD20))

RNA was with nick-translated DNA fragments using conditions described by Margulies, D. H. et al. (Nature, 295: 168 (1982)) or with ^{32}P-labelled oligonucleotide using 4xSSC, 10X Denhardt's, 100 ug/ml salmon sperm DNA at 37°C overnight, followed by washing in 4xSSC at 37°C.

cDNA Preparation and Cloning

Oligo-dT primed cDNA libraries were prepared from poly(A)$^+$ RNA from GM1500 and GM2146 cells by the methods of Land, H. et al. (Nucl. Acids Res., 9: 2251 (1981)) and Gubler, V. and Hoffman, B. J., Gene, 25: 263 (1983), respectively. The cDNA libraries were screened by hybridization (Maniatis, T., supra) with ^{32}P-labelled oligonucleotides using the procedure of de Lange et al. (Cell, 34: 891 (1983)), or with nick-translated DNA fragments.

Oligonucleotide Primer Extension and Cloning

Poly(A)$^+$ RNA (20 ug) was mixed with 1.2 ug primer in 40 ul of 64mM KCl. After denaturation at 90°C for 5 min. and then chilling in ice, 3 units Human Placental Ribonuclease Inhibitor (BRL) was added in 3 ul of 1M Tris-HCl, pH 8.3. The oligonucleotide was annealed to the RNA at 42°C for 15 minutes, then 12 ul of .05M DTT, .05M $MgCl_2$, and 1 mM each of dATP, dTTP, dCTP, and dGTP was added. 2 ul of alpha-^{32}P-dATP (400 Ci/mmol, New England Nuclear) was added, followed by 3 ul of AMV reverse transcriptase (19 units/ul, Life Sciences).

After incubation at 42°C for 105 min., 2 ul 0.5 M EDTA and 50 ul 10mM Tris, 1mM EDTA, pH 7.6 were added. Unincorporated nucleotides were removed by Sephadex G-50 spin column chromatography, and the RNA-DNA hy-

EXPERIMENTAL

Materials and Methods

Tissue Culture Cell Lines

The human cell lines GM2146 and GM1500 were obtained from the Human Mutant Cell Repository (Camden, New Jersey) and cultured in RPMI1640 plus 10% fetal bovine serum (M. A. Bioproducts). The cell line Sp2/0 was obtained from the American Type Culture Collection and grown in Dulbecco's Modified Eagle Medium (DMEM) plus 4.5 g/l glucose (M. A. Bioproducts) plus 10% fetal bovine serum (Hyclone, Sterile Systems, Logan, Utah). Media were supplemented with penicillin/streptomycin (Irvine Scientific, Irvine, California).

Recombinant Plasmid and Bacteriophage DNAs

The plasmids pBR322, pL1 and pUC12 were purchased from Pharmacia P-L Biochemicals (Milwaukee, Wisconsin). The plasmids pSV2-neo and pSV2-gpt were obtained from BRL (Gaithersburg, Maryland), and are available from the American Type Culture Collection (Rockville, Maryland). pHu-gamma-1 is a subclone of the 8.3 Kb HindIII to BamHI fragment of the human IgG1 chromosomal gene. An isolation method for of the human IgG1 chromosomal gene is described by Ellison, J. W. et al., Nucl. Acids Res., 10: 4071 (1982). M8alphaRX12 contains the 0.7 Kb XbaI to EcoRI fragment containing the mouse heavy chain enhancer from the J-C intron region of the M603 chromosomal gene (Davis, M. et al., Nature, 283:733, 1979) inserted into M13mp10. DNA manipulations involving purification of plasmid DNA by buoyant density centrifugation, restriction endonuclease digestion, purification of DNA fragments

can occur by using either the same or different plasmids in the same host.

POLYPEPTIDE PRODUCTS

The invention provides "chimeric" immunoglobulin chains, either heavy or light. A chimeric chain contains a constant region substantially similar to that present in a natural human immunoglobulin, and a variable region having the desired antigenic specificity of the invention, i.e., to the specified human B cell surface antigen.

The invention also provides immunoglobulin molecules having heavy and light chains associated so that the overall molecule exhibits any desired binding and recognition properties. Various types of immunoglobulin molecules are provided: monovalent, divalent, molecules with chimeric heavy chains and non-chimeric light chains, or molecules with the invention's variable binding domains attached to moieties carrying desired functions.

Antibodies having chimeric heavy chains of the same or different variable region binding specificity and non-chimeric (i.e., all human or all non-human) light chains, can be prepared by appropriate association of the needed polypeptide chains. These chains are individually prepared by the modular assembly methods of the invention.

USES

The antibodies of the invention having human constant region can be utilized for passive immunization, especially in humans, without negative immune reac-

lies upon the integration of the desired gene sequences into the host cell genome. Cells which have stably integrated DNA can be selected by simultaneously introducing drug resistance genes such as E. coli gpt (Mulligan, R. C. and Berg, P., Proc. Natl. Acad. Sci., USA, 78: 2072 (1981)) or Tn5 neo (Southern, P. J. and Berg, P., J. Mol. Appl. Genet., 1: 327 (1982)). The selectable marker gene can be either linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection (Wigler, M. et al., Cell, 16: 77 (1979)). A second class of vectors utilizes DNA elements which confer autonomously replicating capabilities to an extrachromosomal plasmid. These vectors can be derived from animal viruses, such as bovine papillomavirus (Sarver, N. et al., Proc. Natl. Acad. Sci., USA, 79: 7147 (1982)), polyoma virus (Deans, R. J. et al., Proc. Natl. Acad. Sci., USA, 81: 1292 (1984)), or SV40 virus (Lusky, M. and Botchan, M., Nature, 293: 79 (1981)).

Since an immunoglobulin cDNA is comprised only of sequences representing the mature mRNA encoding an antibody protein additional gene expression elements regulating transcription of the gene and processing of the RNA are required for the synthesis of immunoglobulin mRNA. These elements may include splice signals, transcription promoters, including inducible promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H. and Berg, P., Mol. Cell Biol., 3: 280 (1983); Cepko, C. L. et al., Cell, 37: 1053 (1984); and Kaufman, R. J., Proc. Natl. Acad. Sci., USA, 82: 689 (1985).

In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with a host cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as specific genes which are capable of providing phenotypic selection in transformed cells. For example, E. coli is readily transformed using pBR322, a plasmid derived from an E. coli species (Bolivar, et al., Gene, 2: 95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance, and thus provides easy means for identifying transformed cells. The pBR322 plasmid or other microbial plasmids must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins. Those promoters most commonly used in recombinant DNA construction include the beta-lactamase (penicillinase) and lactose (beta-galactosidase) promoter systems (Chang et al., Nature, 275: 615 (1978); Itakura et al., Science, 198:1056 (1977)); and tryptophan promoter systems (Goeddel et al., Nucleic Acids Research, 8: 4057 (1980); EPO Publication No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized.

For example, a genetic construct for any heavy or light chimeric immunoglobulin chain can be placed under the control of the leftward promoter of bacteriophage lambda (P_L). This promoter is one of the strongest known promoters which can be controlled. Control is exerted by the lambda repressor, and adjacent restriction sites are known.

(4) A portion of the sequence, frequently the first 6 to 20 codons of the gene sequence may be modified to represent preferred yeast codon usage.

(5) The chimeric genes are placed on plasmids used for integration into yeast chromosomes.

The following approaches can be taken to simultaneously express both light and heavy chain genes in yeast.

(1) The light and heavy chain genes are each attached to a yeast promoter and a terminator sequence and placed on the same plasmid. This plasmid can be designed for either autonomous replication in yeast or integration at specific sites in the yeast chromosome.

(2) The light and heavy chain genes are each attached to a yeast promoter and terminator sequence on separate plasmids containing different selectable markers. For example, the light chain gene can be placed on a plasmid containing the trpl gene as a selectable marker, while the heavy chain gene can be placed on a plasmid containing ura3 as a selectable marker. The plasmids can be designed for either autonomous replication in yeast or integration at specific sites in yeast chromosomes. A yeast strain defective for both selectable markers is either simultaneously or sequentially transformed with the plasmid containing the light chain gene and with the plasmid containing the heavy chain gene.

Another advantage of using cDNA cloning method is the ease and simplicity of obtaining variable region gene modules.

The terms "constant" and "variable" are used functionally to denote those regions of the immunoglobulin chain, either heavy or light chain, which code for properties and features possessed by the variable and constant regions in natural non-chimeric antibodies. As noted, it is not necessary for the complete coding region for variable or constant regions to be present, as long as a functionally operating region is present and available.

Expression vehicles include plasmids or other vectors. Preferred among these are vehicles carrying a functionally complete human constant heavy or light chain sequence having appropriate restriction sites engineered so that any variable heavy or light chain sequence with appropriate cohesive ends can be easily inserted thereinto. Human constant heavy or light chain sequence-containing vehicles are thus an important embodiment of the invention. These vehicles can be used as intermediates for the expression of any desired complete heavy or light chain in any appropriate host.

One preferred host is yeast. Yeast provides substantial advantages for the production of immunoglobulin light and heavy chains. Yeasts carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies now exist which utilize strong promoter sequences and high copy number plasmids which can be used for overt production of the desired proteins in yeast. Yeast re-

may, for example, utilize chemical gene synthesis to generate the UIG probes for the cloning and modification of V regions from immunoglobulin mRNA. On the other hand, oligonucleotides can be synthesized to recognize individually, the less conserved 5'-region of the J regions as a diagnostic aid in identifying the particular J region present in the immunoglobulin mRNA.

A multi-step procedure is utilized for generating complete V+C region cDNA clones from the hybridoma cell light and heavy chain mRNAs. First, the complementary strand of oligodT-primed cDNA is synthesized, and this double-stranded cDNA is cloned in appropriate cDNA cloning vectors such as pBR322 (Gubler and Hoffman, Gene, 25: 263 (1983)). Clones are screened by hybridization with UIG oligonucleotide probes. Positive heavy and light chain clones identified by this screening procedure are mapped and sequenced to select those containing V region and leader coding sequences. In vitro mutagenesis including, for example, the use of UIG oligonucleotides, is then used to engineer desired restriction enzyme cleavage sites. We used this approach for the chimeric 2H7 light chain.

An expedient method is to use synthetic UIG oligonucleotides as primers for the synthesis of cDNA. This method has the advantage of simultaneously introducing a desired restriction enzyme site, such as BstEII (Figure 3) into a V region cDNA clone. We used this approach for the chimeric 2H7 heavy chain.

Second, cDNA constant region module vectors are prepared from human cells. These cDNA clones are modified, when necessary, by site-directed mutagenesis to place a restriction site at the analogous position

GENETIC PROCESSES AND PRODUCTS

The invention provides a novel approach for the cloning and production of a human/mouse chimeric antibody with specificity to a human B cell surface antigen. The antigen is a polypeptide or comprises a polypeptide bound by the 2H7 monoclonal antibody described in Clark et al. Proc. Natl. Acad. Sci., U.S.A. 82:1766-1770 (1985). This antigen is a phosphoprotein designated (Bp35(CD20)) and is only expressed on cells of the B cell lineage. Murine monoclonal antibodies to this antigen have been made before and are described in Clark et al., supra; see also Stashenko, P., et al., J. Immunol. 125:1678-1685 (1980).

The method of production combines five elements:

(1) Isolation of messenger RNA (mRNA) from the mouse hybridoma line producing the monoclonal antibody, cloning and cDNA production therefrom;

(2) Preparation of Universal Immunoglobulin Gene (UIG) oligonucleotides, useful as primers and/or probes for cloning of the variable region gene segments in the light and heavy chain mRNA from the hybridoma cell line, and cDNA production therefrom;

(3) Preparation of constant region gene segment modules by cDNA preparation and cloning, or genomic gene preparation and cloning;

(4) Construction of complete heavy or light chain coding sequences by linkage of the cloned

FIGURE 7 shows the construction of the light and heavy chain expression plasmids pING2106 (panel a) and pING2101 (panel B). The SalI to BamHI fragment from pING2100 is identical to the SalI to BamHI fragment from pING2012E (see panel C). A linear representation of the circular plasmid pING2012E is shown in panel C. The 6.6 Kb SalI to BamHI fragment contains sequences from pSV2-neo, pUC12, M8alphaRX12, and pL1. The HindIII site in pSV2-neo was destroyed before assembly of pING2012E by HindIII cleavage, fill-in, and religation.

FIGURE 8 shows the structure of several chimeric 2H7-V_H expression plasmids. pING2107 is a gpt version of the light chain plasmid, pING2106. The larger ones are two-gene plasmids. pHL2-11 and pHL2-26 contain both H and L genes, while pLL2-25 contains two L genes. They were constructed by joining an NdeI fragment containing either an H or L gene to partially digested (with NdeI) pING2106.

FIGURE 9 shows a summary of the sequence alterations made in the construction of the 2H7 chimeric antibody expression plasmids. Residues underlined in the 5' untranslated region are derived from the cloned mouse kappa and heavy-chain genes. Residues circled in the V/C boundary result from mutagenesis operations to engineer restriction enzyme sites in this region.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

INTRODUCTION

Generally, antibodies are composed of two light and two heavy chain molecules. Light and heavy chains are divided into domains of structural and functional homology. The variable domains of both the light (V_L) and the heavy (V_H) chains determine recognition and specificity. The constant region domains of light (C_L) and heavy (C_H) chains confer important biological

The use of cDNA sequences is particularly advantageous over genomic sequences (which contain introns), in that cDNA sequences can be expressed in bacteria or other hosts which lack appropriate RNA splicing systems.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 shows the DNA rearrangements and the expression of immunoglobulin mu and gamma heavy chain genes. This is a schematic representation of the human heavy chain gene complex (not shown to scale). Heavy chain variable V region formation occurs through the proper joining of V_H, D and J_H gene segments. This generates an active mu gene. A different kind of DNA rearrangement called "class switching" relocates the joined V_H, D and J_H region from the vicinity of mu constant C region to that of another heavy chain C region (switching to gamma is diagrammed here).

FIGURE 2 shows the known nucleotide sequences of human and mouse J regions. Consensus sequences for the J regions are shown below the actual sequences. The oligonucleotide sequence below the mouse kappa J region consensus sequence is a Universal Immunoglobulin Gene (UIG) oligonucleotide. Note that there are only a few J regions with relatively conserved sequences, especially near the constant regions, in each immunoglobulin gene locus.

FIGURE 3 shows the nucleotide sequences of the mouse J regions. Shown below are the oligonucleotide primers UIG-H and UIG-K. Note that each contains a restriction enzyme site. They can be used as primers for the synthesis of cDNA complementary to the vari-

and eukaryotic cells provides a means for the large scale production of a chimeric human/mouse monoclonal antibody with specificity to a human B cell surface antigen.

The invention provides cDNA sequences coding for immunoglobulin chains comprising a constant human region and a variable, non-human, region. The immunoglobulin chains can be either heavy or light.

The invention provides gene sequences coding for immunoglobulin chains comprising a cDNA variable region of the desired specificity. These can be combined with genomic constant regions of human origin.

The invention provides sequences as above, present in recombinant DNA molecules in vehicles such as plasmid vectors, capable of expression in desired prokaryotic or eukaryotic hosts.

The invention provides hosts capable of producing, by culture, the chimeric antibodies and methods of using these hosts.

The invention also provides individual chimeric immunoglobulin chains, as well as complete assembled molecules having human constant regions and variable regions with a human B cell surface antigen specificity, wherein both variable regions have the same binding specificity.

Among other immunoglobulin chains and/or molecules provided by the invention are:

(a) a complete functional, immunoglobulin molecule comprising:

(i) two identical chimeric heavy chains comprising a variable region with a human B cell surface antigen specificity and human constant region and

Seno, M. et al., Nucleic Acids Research, 11: 719-726 (1983), describe the preparation of a cDNA sequence and recombinant plasmids containing the same coding for part of the variable region and all of the constant region of the human IgE heavy chain (epsilon chain).

Kurokawa, T. et al., ibid, 11: 3077-3085 (1983), show the construction, using cDNA, of three expression plasmids coding for the constant portion of the human IgE heavy chain.

Liu, F. T. et al., Proc. Nat. Acad. Sci., USA, 81: 5369-5373 (September 1984), describe the preparation of a cDNA sequence and recombinant plasmids containing the same encoding about two-thirds of the CH_2, and all of the C_H3 and C_H4 domains of human IgE heavy chain.

Tsujimoto, Y. et al., Nucleic Acids Res., 12: 8407-8414 (November 1984), describe the preparation of a human V lambda cDNA sequence from an Ig lambda-producing human Burkitt lymphoma cell line, by taking advantage of a cloned constant region gene as a primer for cDNA synthesis.

Murphy, J., PCT Publication WO 83/03971 (published November 24, 1983) discloses hybrid proteins made of fragments comprising a toxin and a cell-specific ligand (which is suggested as possibly being an antibody).

Tan, et al., J. Immunol. 135:8564 (November, 1985), obtained expression of a chimeric human-mouse immunoglobulin genomic gene after transfection into mouse myeloma cells.

Jones, P. T., et al., Nature 321:552 (May 1986) constructed and expressed a genomic construct where

chimeric antibodies (see p. 5) and suggests, among the technique's many uses the concept of "class switching" (see p. 6).

Taniguchi, M., in European Patent Publication No. 171 496 (published February 19, 1985) discloses the production of chimeric antibodies having variable regions with tumor specificty derived from experimental animals, and constant regions derived from human. The corresponding heavy and light chain genes are produced in the genomic form, and expressed in mammalian cells.

Takeda, S. *et al.*, *Nature*, *314*: 452 (April 4, 1985) have described a potential method for the construction of chimeric immunoglobulin genes which have intron sequences removed by the use of a retrovirus vector. However, an unexpected splice donor site caused the deletion of the V region leader sequence. Thus, this approach did not yield complete chimeric antibody molecules.

Cabilly, S. *et al.*, *Proc. Natl. Acad. Sci., USA*, *81*: 3273-3277 (June 1984), describe plasmids that direct the synthesis in *E. coli* of heavy chains and/or light chains of anti-carcinoembryonic antigen (CEA) antibody. Another plasmid was constructed for expression of a truncated form of heavy chain (Fd') fragment in *E. coli*. Functional CEA-binding activity was obtained by *in vitro* reconstitution, in *E. coli* extracts, of a portion of the heavy chain with light chain.

Cabilly, S., *et al.*, European Patent Publication 125023 (published November 14, 1984) describes chimeric immunoglobulin genes and their presumptive products

INFORMATION DISCLOSURE STATEMENT*

Approaches to the problem of producing chimeric antibodies have been published by various authors.

Morrison, S. L. et al., Proc. Natl. Acad. Sci., USA, 81: 6851-6855 (November 1984), describe the production of a mouse-human antibody molecule of defined antigen binding specificity, produced by joining the variable region genes of a mouse antibody-producing myeloma cell line with known antigen binding specificity to human immunoglobulin constant region genes using recombinant DNA techniques. Chimeric genes were constructed, wherein the heavy chain variable region exon from the myeloma cell line S107 were joined to human IgG1 or IgG2 heavy chain constant region exons, and the light chain variable region exon from the same myeloma to the human kappa light chain exon. These genes were transfected into mouse myeloma cell lines. Transformed cells producing chimeric mouse-human antiphosphocholine antibodies were thus developed.

Morrison, S. L. et al., European Patent Publication No. 173494 (published March 5, 1986), disclose chimeric "receptors" (e.g. antibodies) having variable regions derived from one species and constant regions derived from another. Mention is made of utilizing cDNA cloning to construct the genes, although no details of cDNA cloning or priming are shown. (see pp 5, 7 and 8).

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* Note: The present Information Disclosure Statement is subject to the provisions of 37 C.F.R. 1.97(b). In addition, Applicants reserve the right to demonstrate that their invention was made prior to any one or more of the mentioned publications.

invention of recombinant DNA cloning. Monoclonal antibodies produced from hybridomas are already widely used in clinical and basic scientific studies. Applications of human monoclonal antibodies produced by human hybridomas hold great promise for the treatment of cancer, viral and microbial infections, certain immunodeficiencies with diminished antibody production, and other diseases and disorders of the immune system.

Unfortunately, a number of obstacles exist with respect to the development of human monoclonal antibodies. This is especially true when attempting to develop therapeutically useful monoclonal antibodies which define human cell surface antigens. Many of these human cell surface antigens are not recognized as foreign antigens by the human immune system; therefore, these antigens are not immunogenic in man. By contrast, human cellular antigens which are immunogenic in mice can be used for the production of mouse monoclonal antibodies that specifically recognize the human antigens. Although such antibodies may be used therapeutically in man, repeated injections of "foreign" antibodies, such as a mouse antibody, in humans, can lead to harmful hypersensitivity reactions as well as increased rate of clearance of the circulating antibody molecules so that the antibodies do not reach their target site. Furthermore, mouse monoclonal antibodies may lack the ability to efficiently interact with human effector cells as assessed by functional assays such as antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis (CDC).

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